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Cloning and expression of aequorin genes from jellyfish *Aequorea* and characterization of aequorins activities*

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Abstract —— Two new aequorin genes, *aeqxm* and *aeqxxm*, were isolated from jellyfish *Aequorea macrodactyla* and *Aequorea parva* respectively, which are commonly found in the warmer waters on the coastal region of the East China Sea. The DNA sequences of the two genes have no introns and each one contains an ORF of 585 bp in full-length encoding a 195-aa protein. The two genes of *aeqxm* and *aeqxxm* share nucleotide homologies of 80.7% and 85.1% with AEVAQ440X respectively, and the corresponding proteins share amino acid homologies of 84.7% and 84.2% with AEVAQ440X. High amino acid homology was found between apoaeqxm and apoaeqxxm. The two genes were cloned into expression vector pTO-T7 respectively, and the expression yields amounted to 40% of the total protein in *E. coli* BL21. The activities of the two photoproteins were reconstituted by incubating the expressed apoproteins with coelenterazine f. In the presence of Ca ion, both of the regenerated *aeqxm* and *aeqxxm* exhibited an emission peak at the wave length of 470 nm.

Key words *Aequorea macrodactyla*, *Aequorea parva*, aequorin, expression

INTRODUCTION

Aequorin is a bioluminescent protein, isolated from jellyfish *Aequorea*. The aequorin complex consist of a 22 000 Mr apoaequorin protein, molecular oxygen and the luminophore coelenterazine. When three Ca ions bind to this complex, coelenterazine is oxidized to coelenteramide, with aconcomitant release of carbon dioxide and blue light (emission maximum~469nm). As light emission from aequorin is sensitive and specific to Ca^{2+}, the protein provides an excellent method for the monitoring and measuring of Ca^{2+}, concentration. The photoprotein was introduced into bacteria, yeast, plant and animal cells to monitor events inside

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living cells or organisms (Salar-Newby *et al.*; 1998; Hampton *et al.*, 1998; Cessna *et al.*, 1998; Leung *et al.*, 1998). As a good marker of gene expression *in vivo* and luminent immunoassay, aequorin has also been applied to quantitative PCR based on a dual-analyte chemiluminescence by hybridization assay for target DNA and internal standard (Casadei *et al.*, 1990; Zenno and Inouye, 1990; Verhaegen and Christopoulos, 1998; Actor *et al.*, 1998).

The cDAN-AQ440 (AEVAQ440X) for apoaequorin has been isolated from *Aequorea victoria* by Inouye *et al.* (1985; 1986). The primary structure has also been determined by sequencing the protein by Charbonneau *et al.* (1985). The mature apoaequorin is composed of 189 amino acid residues, and has three EF hand structures that are characteristic of Ca^{2+}-binding sites. In the same year, Prasher *et al.* (1985) cloned the cDNA of apoaequorin from *Aequorea victoria* and expressed the protein in *E. coli*. The sequences of the two cDNAs are different in 52 nucleotide sites. Among 9 sequences of aequorin in Genbank, six are isolated from *Aequorea victoria* and one from jellyfish unknown, and two are artificial sequences. High nucleotied homology of above 90% was found among the six sequences from *Aequorea victoria*.

We have isolated new aequorin genes —— *aeqxm* and *aeqxxm* from fellyfish *Aequorea macrodactyla* and *Aequorea parva* respectively, and expressed the apoproteins in *E. coli*. The expressed apoproteins have normal bioactivities.

MATERIALS AND METHODS

Aequorea macrodactyla and Aequorea parva

The jellyfish *Aequorea macrodactyla* and *Aequorea parva* were collected from the coastal regions of the East China Sea near Xiamen.

Bacterial strains and plasmids

The *E. coli* strains used were JM101 and BL21 (DE3). The plasmids employed were pTO-T7. pTO-T7EGFP (Luo *et al.*, 2000) and pEGFP and pGFPUV purchased from Clontech.

Enzymes and chemicals

All restriction endonuclease, *E. coli* T4 DNA lingase were purchased from Promega, USA. NBT/BCIP and Gel extraction mini kit were purchased from Watson, Shanghai, China. DIG Labeling Mix was purchased from Beohringed Mannbein GmbH, Germany. Taq enzyme and dNTP were purchased from Sangon, Shanghai, China. Coelenterate f was purchased from Molecular Probes, USA. PCR primers (see Table 1) were synthesized by Bioasia, Shanghai, China.

Table 1. PCR primers and the sequences

Primers	Sequences
aqF448	5′- ATC CGT ATA TGG GGT GAT GC- 3′
aqR712	5′- GAG CTT CTT AGG GGA CAG CT- 3′
aqF93	5′- GTC TCG ACA ACA AGC AAA C- 3′
aqF1	5′- CTG CAG TGA ATT CAT GAC CAG CA ATA CGC- 3′
aqR1	5′- CTC GAG TTA GGG GAC AGC TCC- 3′

Preparation of total DNA from Aequorea

The outer margin of the umbrella of the jellyfish was collected. Two outer margins were ground in a homogenizer with 4 cm^3 of DNA extraction solution (20 mmol·Tris-HCl pH 7.4, 10 mmol/dm^3 EDTA, 1% SDS). After that had been extracted with equal volume of phenol saturated, the upper aqueous phase was digested with 5 mm^3 of RNase A (10 mg/cm^3) in 37℃ for 2 hours and the mixture was extracted with chloroform and finally the upper aqueous phase was kept. DNA was precipitated with absolute ethanol and 0.1 mol sodium acetate (pH5), collected by centrifugation at 12 000 rpm for 10 min, and dissolved in 10 mmol/dm^3 Tris-HCl, pH 7.5/1.0 mmol/dm^3 EDTA to give a concentration of 0.1 mg/cm^3, and stored at - 20℃.

Preparation of probe and Southern hybridization

Two specific primers aqF448 (5′- ATC CGT ATA TGG GGT GAT GC-3′) / apR712 (5′- GAG CTT CTT AGG GGA CAG CT- 3′) were designed and synthesized according to the mucleotide sequence of AEVAQ440X. A fragment of 264bp was amplified by PCR with DNA of *Aequorea parva* as template, and sequenced. The fragment shared a nucleotide homology of 87.5% with the cDNA of AEVAQ440X. The 264bp fragment was labelled with DIG by PCR with DIG labelled dNTP Mix, and used as probe in Southern hybridization. The operations of Southern hybridization and dot bloting were carried out according to standard methods (Sanbrook *et al.*, 1989).

Expression of apoaequorins in E. coli

The transformed *E. coli* strains BL21 (DE3) carrying the expression plasmid were grown in 200 cm^3 of LB medium (Kan, 100 μg/cm^3), Incubations were carried out in a shaker at 37℃. When the OD600 of the culture medium reached about 0.8, IPTG was added to a final concentration of 0.2 mmol/cm^3, and the incubation was continued at 20℃ for 6 hours. The cells were harvested by centrifugation and resuspended in 10 cm^3 of 20 mmol/dm^3 Tris-HCl (pH 7.6). Cell disruption was achieved by sonication (8 × 30s, in an ice bath) with a Sonics & Materials INC Danbury Connecticut Model Sonifier. After centrifugation at 15 000 rpm for 10 min in a 3K18 Model refrigerated centrifuge (Sigma), the precipitant was dissolved in 20 mmol/dm^3 Tris-HCl (pH 7.6) to give a volume equal to the supernatant.

Twenty-five mm^3 of supernatant and precipitant solution respect was performed with SDS PAGE. The concentrations of stacking Gels and resolving Gels were 5% and 12% respectively.

Assay for aequorin activity (Shimomura and Johnson, 1975)

Aequorin was regenerated from apoaequorin by adding 10 mm^3 of 2-ME and 6 μg of coelenterazine f (1 μg/mm^3, in absolute methyl alcohol) to 1.0 cm^3 of the extract and the mixture was allowed to stand for 3 hours in an ice bath. The mixture was placed in fluorescent parorama and injected with 1.5 cm^3 of 30 mmol/dm^3 $CaCl_2$/30 mmol/dm^3 Tris-HCl, pH 7.6. The maximal light intensity was recorded as a measure of aequorin activity.

Analysis of DNA and amino acid sequence

The software dnasis was used to analyse the ORF of DNA sequence and dnastar was used to analyse the homology of amino acid sequence of protein.

Genbank accession number

AEVAQ440X L29571, *aeqxm* AY013823, *aeqxxm* AY013822

RESULTS

Isolation of aeqxm gene

Aequorin gene from *Aequorea macrodactyla* was isolated by the method of combination of PCR amplification and Southern hybridization. Six specific primers were designed and synthesized according to the nucleotide sequence of AEVAQ440X gene. PCR amplifications were carried out under different conditions with DNA of *Aequorea macrodactyla* as template by several primer pairs composed of three sense primers and three antisense primers. The positive fragments were selected by Southern hybridization and sequenced. Finally, a positive fragment was selected from the PCR products by primers aqF93/aqR712.

The fragment was cloned into pMD-18T vector to obtain plasmid pTAxm positive in dot bloting. Sequencing result shows that the fragment is *aeqxm* gene (see Fig. 1). The DNA sequence of *aeqxm* has no introns and contains an ORF of 585bp in full-length, encoding an 195-aa protein with an Mr of about 23 kD (Genbank accession No: AY013823). The nucleotide homology between *aeqxm* and AEVAQ440X is 80.7%.

Isolation of aeqxxm gene

Aequorin gene from *Aequorea parva* was isolated by the same method. Specific primers aqF1/aqR1 were designed and synthesized according to the nucleotide sequence of *aeqxm* gene. PCR amplifications were carried out under different conditions. A positive fragment was selected from the PCR products by Southern hybridization.

The fragment was cloned into pMD-18T vector to obtain plasmid pTAxxm positive in dot bloting. Sequencing result shows that the fragment is *aeqxxm* gene (see Fig. 2). The DNA sequence of *aeqxxm* also has no introns and contains an ORF of 585bp in full-legth, encoding a 195-aa protein (Genbank accession No: AY013822). *Aeqxxm* has the nucleotide homology of 85.1% and 87.2% to AEVAQ440X and *aeqxm* respectively.

```
   1 ATGACCAGCAAATACGCCGTCAAACTTGAGCCAGACTTTGAGAACCCAAAATGGGTTGGT
     M  T  S  K  Y  A  V  K  L  E  P  D  F  E  N  P  K  W  V  G
  61 CGACACAAGCATATGTTCAAATTCCTTGATGTCAATCAAAATGGAAAGATCTCTCTTGAC
     R  H  K  H  M  F  K  F  L  D  V  N  Q  N  G  K  I  S  L  D
 121 GAGATGGTCTACAAGGCGTCCGACATTGTCATCAACAATCTTGGGGCGACACCCGAACAA
     E  M  V  Y  K  A  S  D  I  V  I  N  N  L  G  A  T  P  E  Q
 181 GCTAAACGACACAAGGACGCCGTAGAGGCTTTCTTCGGAGGCGCCGGAATGAAATACGGC
     A  K  R  H  K  D  A  V  E  A  F  F  G  G  A  G  M  K  Y  G
 241 GTGGAAACTGAATGGCCTGAATACATCGAAGGATGGAAGAATTTGGCGAGAACGGAATTA
     V  E  T  E  W  P  E  Y  I  E  G  W  K  N  L  A  R  T  E  L
 301 GACAGATTTGCAAAGAATCAAATAACGCTCATTCGCTTGTGGGGCGATGCGTTGTTTGAC
     D  R  F  A  K  N  Q  I  T  L  I  R  L  W  G  D  A  L  F  D
 361 ATCATTGACAAAGATCAAAATGGTGCTATCACCTTGGACGAATGGAAGAAATACACACTG
     I  I  D  K  D  Q  N  G  A  I  T  L  D  E  W  K  K  Y  T  L
 421 TCAGCTGGCATCATTCAGTCAGCAGAAGATTGCGAGATAACGTTCAAGGTATGTGATTTG
     S  A  G  I  I  Q  S  A  E  D  C  E  I  T  F  K  V  C  D  L
 481 GACGACAGTGGAAGACTTGATGCCGACGAAATGACACGACAACACATCGGATTTTGGTAC
     D  D  S  G  R  L  D  A  D  E  M  T  R  Q  H  I  G  F  W  Y
 541 ACCATGGATCCGGCGTGCGAAAAGCTCTACGGAGGAGCTGTCCCCTAA
     T  M  D  P  A  C  E  K  L  Y  G  G  A  V  P  *
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Fig. 1. DNA sequence and deduced amino acid sequence of *aeqxm*.

Analysis of amino acid sequences predicted from aeqxm and aeqxxm

AEVAQ440X encodes 196 amino acid residues while both of *aeqxm*, *aeqxxm* encode 195 amino acid residues lacking a Gln in the fifth amino acid residue. AEVAQ440X share amino acid homology of 84.7% and 87.2% to apoaeqxm, apoaeqxxm respectively. The high amino acid homology of 94.4% was found between apoaeqxm and apoaeqxxm (see Fig. 3).

Because the chemical analysis of whole apoaequorin protein shows that the sequence started from the eighth amino acid residue from the first methionine, Inouye *et al*. defined the eighth amino acid residue encoded by AEVAQ440X as Position 1 and located three possible Ca^{2+}-binding sites at Positions 24~35, 117~128 and 153~164. We predicted that the three possible Ca^{2+}-binding sites of *aeqxm* and *aeqxxm* were at Positions 30~41, 123~134 and 159~170. While comparing the amino acid sequences of the three Ca^{2+}-binding sites of *aeqxm* and

aeqxxm with that of AEVAQ440X, there were one amino acid residue difference in the first Ca^{2+}-binding site and three in the third Ca^{2+}-binding site. But they were exactly the same in the second Ca^{2+}-binding site.

Expression of apoaeqxm and apoaeqxxm in E. coli

The DNA fragments of *aeqxm* and *aeqxxm* were cut from plasmids pTAxm. pTAxxm with EcoRI/XhoI, then cloned into expression vector pTO-T7 (Luo *et al.*, 2000) to obtain expression plasmids pTO-T7Axm and pTO-T7Axxm (see Fig. 4). Apoaexm and apoaeqxxm were expressed in the transformed *E. coli* strains BL21 (DE3) carrying the plasmids pTO-T7Axm and pTO-T7Axxm respectively. As a result (see Fig. 5), the expressed apoaeqxm and apoaeqxxm amounted to about 40% of the total protein, with about 70% of which being found in the cyosol.

```
  1 ATGACCAGCAAATACGCAGTCAAGCTCGAACCAGACTTTGAGAATCCAAGATGGATTGGT
    M  T  S  K  Y  A  V  K  L  E  P  D  F  E  N  P  R  W  I  G
 61 CGACACAAACATATGTTCAACTTTCTTGATGTCAACCAAAATGGAAAGATCTCTCTTGAC
    R  H  K  H  M  F  N  F  L  D  V  N  Q  N  G  K  I  S  L  D
121 GAAATGGTCTACAAGGCGTCTGATATTGTCATAAACAATCTTGGAGCAACACCTGAGCAA
    E  M  V  Y  K  A  S  D  I  V  I  N  N  L  G  A  T  P  E  Q
181 GCAAAACGCCACAAGGAGGCTGTAGAAGCTTTCTTTGGAGGAGCTGGTATGAAATATGGT
    A  K  R  H  K  E  A  V  E  A  F  F  G  G  A  G  M  K  Y  G
241 GTAGAAACCGAATGGCCTGAATACATCAAAGGATGGAAGAAATTGGCTCAAACAGAATTA
    V  E  T  E  W  P  E  Y  I  K  G  W  K  K  L  A  Q  T  E  L
301 GACAAATTTGCAAAGAATCAAGTAACCCTCATCCGTTTATGGGGTGATGCTTTGTTAGAT
    D  K  F  A  K  N  Q  V  T  L  I  R  L  W  G  D  A  L  L  D
361 ATCATTGACAAAGATCAGAATGGTGCAATCACATTGGACGAGTGGAAGAAATACACACAA
    I  I  D  K  D  Q  N  G  A  I  T  L  D  E  W  K  K  Y  T  Q
421 TCAGCTGGTATCATTCAATCAGCAGAAGATTGTGAGGAAACATTCAAAGTGTGTGATCTG
    S  A  G  I  I  Q  S  A  E  D  C  E  E  T  F  K  V  C  D  L
481 GATGACAGTGGCCGACTAGATGCTGATGAGATGACACGACAACATATAGGATTTTGGTAC
    D  D  S  G  R  L  D  A  D  E  M  T  R  Q  H  I  G  F  W  Y
541 ACCATGGATCCTGCTTGTGAAAAGCTCTACGGAGGAGCTGTCCCCTAA
    T  M  D  P  A  C  E  K  L  Y  G  G  A  V  P  *
```

Fig. 2. DNA sequence and deduced amino acid sequence of *aeqxxm*.

Assay for bioactivities of aeqxm and aeqxxm (Shimomura and Johnson, 1975)

Aeqxm and *aeqxxm* were regenerated from apoaeqxm and apoaeqxxm respectively by adding 2-ME and coelenterzine f to 1.0 cm^3 of the extract. The mixture was allowed to stand for 3 hours in an ice bath, then placed in fluorescent panorama and injected with 1.5 cm^3 of 30

mmol/ dm^3 $CaCl_2$/ 30 mmol/ dm^3 Tris-HCl, pH 7.6. That the resulting products emitted blue light at the peak wave length of 470 nm show that the expressed apoaeqxm and apoaeqxxm have normal biofunctions.

The activities of *aeqxm* and *aeqxxm* increased along with the time of regeneration and reached the best level after 3 hours of incubation. *Aeqxm* shows good activity after 30 minutes incubation and the value is one time higher than that of *aeqxxm* after 3 hours of incubation (data not shown). *Aeqxm* activity reached the maximum under the condition of 0 mol/ dm^3 NaCl, pH 7.6 and 30℃ below. *Aeqxxm* activity reached the maximum under the condition of 0 mol/ dm^3 NaCl, pH 7.6~ 8.5 and 30℃ below.

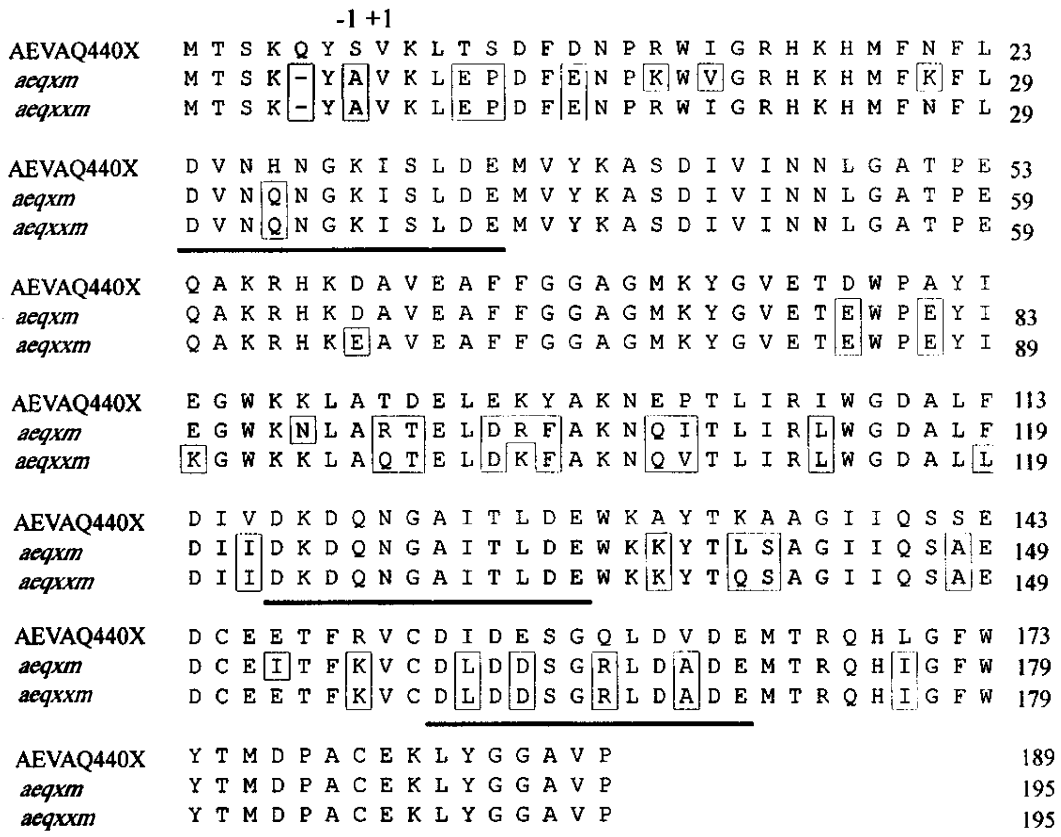

Fig. 3. Comparison of the amino acid sequences of AEVAQ440X, *aeqxm* and *aeqxxm*. Black bars show the Ca^{2+}-binding site. The mature apoaequorin of AEVAQ440X starts from the eighth amino acid residue Val. Box residues differ from AEVAQ440X.

DISCUSSION

By the method of combination of PCR amplification and Southern hybridization, we isolated the genomic DNA sequences of new aequorin genes — *aeqxm* from *Aequorea macrodactyla*, *aeqxxm* from *Aequorea parva* in the East China Sea, and expressed apoaeqxm and

apoaeqxxm with biological activities in *E. col*.

The known nucleotide sequences of apoaequorins are all cDNA sequences, and there are no reports on the corresponding genomic sequences. The nucleotide sequences of *aeqxm* and *aeqxxm* were isolated from genomic DNA of *Aequorea* and both contained an ORF of 585bp without introns. The two genes of *aeqxm* and *aeqxxm* shared nucleotide homologies of 80.7% and 85.1% to AEVAQ440X respectively, and the nucleotide homology between *aeqxm* and *aeqxxm* is 87.2%. AEVAQ440X gene encoded a 196-aa protein, while both *aeqxm* and *aeqxxm* genes encoded a 195-aa protein lacking a Gln in the 5th site. Apoaeqxm and apoaeqxxm shared amino acid homologies of 84.7% and 84.2% to AEVAQ440X respectively and shared high amino acid homology of 94.4% to each other.

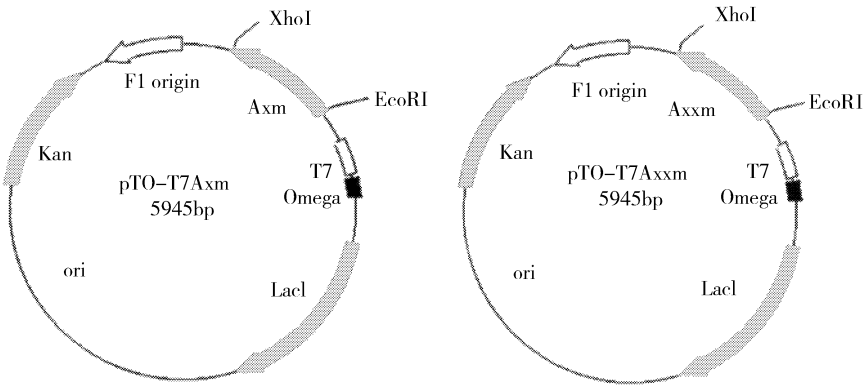

Fig. 4. Plasmids of pTO-T7Axm, pTO-T7Axxm.

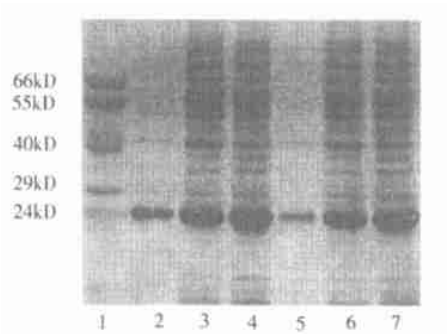

Fig. 5. SDS-PAGE of expressed apoaeqxm and apoaeqxxm in *E. coli*.
1. Molecular marker, 2. apoaeqxm in the pellet, 3. apoaeqxm in the cytosol, 4. expressed apoaeqxm in *E. coli*, 5. apoaeqxxm in the pellet, 6. apoaeqxxm in the cytosol and 7 expressed apoaeqxxm in *E. coli*.

Cormier *et al*. (1989) observed five aequorin isotypes extracted from *Aequorea* tissue on 2-dimensional gels. The multiple isotypes could be caused by the presence of a multi-gene family since Southern blot analysis of *Aequorea* DNA suggests the presence of a minimum of four aequorin genes. We isolated aequorin genes from *Aequorea macrodactyla* and *Aequorea par-*

va, and further study is required to determine the presence of a multi-gene family in these two species of *Aequorea*. The isolation and expression of *aeqxm* and *aeqxxm* will enhance the homology research of nucleotide sequence and protein structure of aequorins and facilitate to find out the essential conservative element, and provide informations about aequorin evolution.

To enhance the wide application of aequorin, aequorin from *Aequorea victoria* was modified to obtain some mutants such as increasing biological activity. The requirement for a proline residue at the C-terminus was investigated by measuring luminescence activities of a series of C-terminal deleting mutants, substitution mutants and an addition mutant. Modified apoaequorin genes coding for apoaequorin by cassette mutation altered the bioluminescent activity. The recombinant aequorin and recombinant semi-synthetic aequorins are highly suited for monitoring cellular Ca^{2+} (Nomura *et al.*, 1991; Shimomura *et al.*, 1990; Kurose *et al.*, 1989).

Kurose *et al.* (1989) used the technique of site-specific mutagenesis to replace the three cysteine residues with serine and found that modified aequorin with all three cysteines replaced by serine had luminescence activity equal to or greater than that of the wild-type aequorin. Prasher *et al.* made genetic mutations in the second Ca^{2+}-binding site of aequorin, and found that specific examples of modified apoaequorins possess greater bioluminescent activity than unmodified apoaequorin, when aspartate 124 is changed to serine, glutamate 135 is changed to serine, or glycine 129 is changed to alanine (United States Patent 5360728, in 1994). The amino acid sequences coded by *aeqxm* and *aeqxxm* are the same as AEVAQ440X in those sites, so similar mutations can be made to enhance the bioluminescent activity of *aeqxm* and *aeqxxm*.

Aeqxm and *aeqxxm* with normal biofunctions may be used as good markers of gene expression and sensitive Ca^{2+} indicators in living cells. The isolation and expression of two new aequorin genes will provide abundant resources for aequorin modification. and make it possible to apply aequorin to a wide variety field of biological research.

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